RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF PARACETAMOL, ACECLOFENAC AND TRAMADOL INCOMBINED DOSAGE FORM

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Submitted by

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1. INTRODUCTION

Analytical chemistry is a branch of chemistry that deals with the separation, identification and determination of components in a sample. It is the science of making quantitative measurements, which requires background knowledge of chemical and physical concepts.

Analytical chemistry may be defined as the science and art of determining the composition of material in terms of elements or compounds contained in it.

For analysis of these drugs different analytical methods are routinely being used. These analytical methods are classified as classical and instrumental.

- The classical methods include Gravimetric and Titrimetric.
- These methods are simple but less precise and more time consuming so Now a days these methods are not suggested for the routine analysis.
- The instrumental methods include electrical methods -voltametry, Coulometry and optical methods - absorption and emission methods.
- The absorption methods include visible spectrophotometry, ultraviolet spectrophotometry, infrared spectrophotometry, atomic absorption spectrophotometry and emission methods include emission spectroscopy, flame photometry, fluorimetry, etc.
- The other prominent methods include isotopes, radioactivity, x-ray fluorescence and separation methods as various chromatographic principles viz. HPLC, GC and HPTLC etc.

These methods like HPLC,Spectrophotometry are easy to perform, precise and show reproducible results as compared to any other methods.

Importance of Analytical Methods

The newly developed analytical methods having their importance in different fields.

- Research & Development Centre
- Quality control Department
- Approved Testing Laboratories
- Chemical Analysis Laboratories

Chromatography Techniques

Classification of Chromatography Techniques

I] According to the nature of stationary and mobile phase

- Gas Solid Chromatography
- Gas Liquid Chromatography
- Solid Liquid Chromatography
- Liquid Liquid Chromatography

II] According to mechanisms of separation, chromatographic methods are divided into following general area

- Adsorption chromatography
- Partition chromatography
- Size exclusion chromatography
- Ion exchange chromatography

Adsorption chromatography, the analytes interact with solid stationary surface and are displaced with the eluent for active sites on surface.

Partition chromatography, results from a thermodynamic distribution between two liquid (or liquid like) phase. On the basis of relative polarities of stationary and mobile phases. Partition chromatography can be divided into ***normal phase and reverse- phase chromatography***

Size-exclusion Chromatography, involves a solid stationary phase with controlled pore size. Solutes are separated according to molecular size, with the large molecules unable to enter the pores elute first.

Ion-exchange Chromatography, involves a solid stationary phase with anionic or cationic groups on the surface to which solute molecules of opposite charges are attracted.

In chromatographic separation, HPLC and HPTLC methods have widely been exploited in pharmaceutical analysis because of its simplicity, precision, accuracy and reproducibility of results.

HPLC

High Performance Liquid Chromatography (HPLC)

In **gradient** system eluent composition and strength is steadily changed during the run.

Table No: 1 Validation parameters as per ICH guidelines and USP

1. Specificity	5. Limit of Detection
2. Linearity & Range	6. Limit of Quantitation
3. Accuracy	7. Robustness
4. Precision	8. System Suitability

Specificity

The determination of method specificity can be achieved in two ways, first and most desirable, all potential interfering compounds can be tested to demonstrate their separation from the peak (S) of interest with a specified resolution (usually $R_S \geq 2$) A second method for achieving a specificity is the use of selective detectors especially for co eluting compounds. For example a selective detectors (e.g. electrochemical and radioactivity) will respond some compounds but not to others**.**

Linearity

The linearity of a method is to measure a calibration plot of area Vs concentration approximates a straight line. Linearity can be assessed by performing single measurements at several analyte concentrations. The data are then processed using a linear least-squares regression. The resulting plot slope, intercept, and correlation coefficient provide the desired information on linearity.

The numerical value of the slope and intercept will depend on the response measured, but intercepts greater than 2% (relative to the target level response) are typically expected with well-designed HPLC methods for major component analysis. This approach involves determining the response factor (or sensitivity) Vs

analyte concentration (or log concentration for a wide range). This response factor (RF) is calculated as

$$RF = DR/C$$

Where DR is the detector response (peak area or peak height) and C is the concentration of the analyte.

Range

The range of method can be defined as the lower and upper concentrations for which the analytical method has adequate accuracy, precision, and linearity. The range of concentrations examined will depend on the type of method and its use.

Accuracy

A sample (whose "true value" is known) is analyzed and the measured value should ideally be identical to the true value with high accuracy,. Typically, accuracy is represented and determined by recovery studies, but there are three ways to determine accuracy:

1) Comparison to a reference standard

2) Recovery of the analyte spiked into blank matrix, or

3) Standard addition of the analyte.

Accuracy determination for an HPLC method should be carried out with a minimum of nine measurements using at least three concentrations. This approach

minimizes any variability and/or bias in sample preparation technique and analysis for one sample at only one concentration.

Precision

Repeatability, intermediate precision and reproducibility.

- **Repeatability**

Repeatability is the precision of a method under same operating conditions over a short period of time. One aspect of this is instrument precision. This is measured by the sequential, repetitive injection of the same homogenous sample (typically, 10 or more times), followed by the averaging of the peak area or peak-height values and determination of the relative standard deviation of all injections. A second aspect is sometimes termed intra-assay precision and involves multiple measurements of the same sample (different preparation) by the same analyst under the same conditions.

- **Intermediate precision**

It is the agreement of complete measurements (including standards) when the same method is applied many times within the same laboratory. This can include multiple preparations of samples and standards were analysis on different days, instruments. or analysts to confirm reproducibility.

- **Reproducibility**

The precision assessment during initial method validation often applies to the first two of these: repeatability and intermediate precision. Reproducibility is usually

determined during method transfer or crossover to another laboratory or location. Precision often is expressed by the standard deviation (SD) or relative standard deviation (RSD) of a data set. If a set of n measurements is defined as

$$X = \frac{\sum_{i=1}^{n} Xi}{n}$$

Where xi are the individual measurements on the sample . The standard deviation of these data is then

$$SD = \sqrt{\frac{\sum_{i=1}^{n} (X_i - X)^2}{n-1}}$$

And the relative standard deviation (RSD) or coefficient of variation (CV) is

RSD (%) = 100 SD / x

Where SD= Standard deviation, X= average

Limit of Detection and Limit of Quantitation

The Limit of Detection (LOD) can be defined as the smallest level of analyte that gives a measurable response.

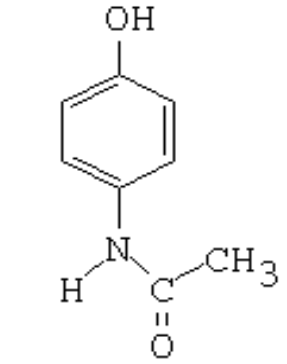
1. DRUG PROFILE OF PARACETAMOL

1.1 Chemical Name **:** N-Acetyl-p-amino phenol; 4-Hydroxyacetanilide; N-(4- hydroxyphenyl) acetamide.

1.2. Molecular Formula **:** $C_8H_9NO_2$

1.3 Molecular Weight **:** 151.2

1.4 Structural Formula **:**



Paracetamol

Fig: 1

1.5. Physicochemical Properties

(a) ***Description and Solubility*****:**

Paracetamol appears as white crystals or white crystalline powder. It is freely soluble in ethanol (95%) methanol, dimethyl formamide, ethylene dichloride, ethyl acetate and in acetone, sparingly soluble in water, very slightly soluble in dichloromethane and in ether, insoluble in petroleum ether, pentane and benzene.

(b) Melting Point : Reported 169°C – 170.5°C

Observed 170 °C

(c) Pka : 9.05 at 25°C.

1.6. Mechanism of action

It acts by inhibiting cyclo-oxygenase (COX) enzyme in brain, thus inhibiting formation of prostaglandins[31]It is a poor inhibitor of PG synthesis in peripheral tissues. The central analgesic action of Paracetamol is that, it raises pain threshold, but has weak peripheral anti inflammatory component.Paracetamol is a god and promptly acting antipyretic.

1.7. Category: Analgesic and anti pyretic.

2. DRUG PROFILE OF ACECLOFENAC

2.1. Chemical Name : [O-(2,6-Dichloroanlino)phenyl]acetate glycolicolic acid ester; 2-(2,6-Dichloroanalino) phenylacetoxyacetic acid.

2.2. Molecular formula : $C_{16}H_{13}Cl_2NO_4$

2.3. Molecular weight : 354.2

2.4. Structural formula:

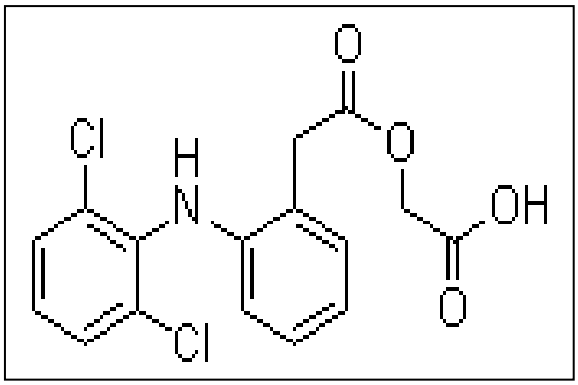


Fig: 2

2.5. Physicochemical Properties:

a) Description and solubility

Appears as white crystalline powder. Practically insoluble in water, soluble in alcohol and in methanol, freely soluble in acetone and in dimethylformamide.

b) Melting point Reported: 149-150.^{0}C

Observed: 149 ^{0}C

2.6. Mechanism of action[28]

It inhibits interleukin PGE and TNF synthesis and stimulates GAG formation. Thus uniquently helps in repair also.

2.7. Category Rheumatoid arthritis,

Osteoarthritis.

3. DRUG PROFILE OF TRAMADOL

3.1 The chemical name : Tramadol hydrochloride is (±) cis-2-[(dimethylamino) methyl]-1-(3-methoxyphenyl cyclohexanol hydrochloride.

3.2.Molecular formula **:** $C_{16}H_{25}NO_2$ HCl.

3.3 Molecular weight **:** 299.8

3.4 Structural Formula:

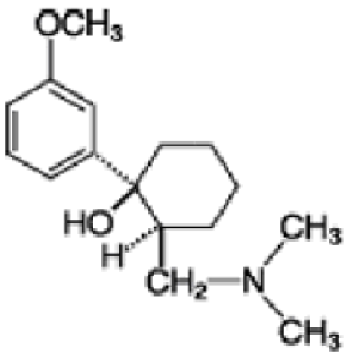


Fig: 3

3.5 Physicochemical Properties:

a) Description and Solubility:

It is a white, bitter, crystalline and odorless powder .Tramadol is readily soluble in water and ethanol, methanol and other organic solvents.

b) Melting point: 171^0 C

3.6. Mechanism of action

Its affinity for μ opoid receptors is modest while that α and β is weak. Unlike other opoids, it inhibits reuptake of NA and 5-HT, and thus activates [30]noaminergic spinal inhibition of pain. Its analgesic action is only partially reversed by opoid antagonist naloxone.

3.7. Category : Arthralgia

Muskulo skeletal pain

Colic's

Post traumatic cases.

2. AIM AND OBJECTIVE

- Validation is a necessary and important step in both framing and documenting the capabilities of the developed method. Most of the reviews on this combination Paracetamol, Aceclofenac and Tramadol are associated with its clinical pharmacology, adverse effects, potential drug interaction and warnings.

- There is no method reported so for the simultaneous estimation of these three drugs from combination formulations. Some studies have been reported recently pertaining to simultaneous estimation of Paracetamol, and Aceclofenac by on pharmaceutical dosage forms by RP-HPLC, and spectroscopic methods. And spectrophotometric methods are reported for Paracetamol, Aceclofenac with Tramadol and for two drug combinations.

- Through this project, an attempt is being made to develop and validate simple, cost effective, sensitive methods for quantitative monitoring of Paracetamol, Aceclofenac and Tramadol in pure as well as tablet dosage forms.

3. REVIEW OF LITERATURE

Shanmugam[41] **et al** developed Spectrophotometric method for the estimation of Aceclofenac in tablets.

Aceclofenac is NSAI and anti-rheumatic drug. A simple accurate, rapid, economical and precise procedure for Spectrophotometric estimation aceclofenac in tablet formulation has been developed. The drug obeys Beer's law in the concentration ranges employed in the method. Shows absorbance maxima at 275 nm methanol and results of the analysis were validated by recovery studies.

4. MATERIALS AND METHODS

4.1 U V Spectroscopic Method

4.1.1 Materials

All The Chemicals and reagents used in the investigation were of analytical grade, which are

- Paracetamol obtained from M/S Biocon Ltd., Bangalore
- Aceclofenac obtained form M/S Ontop Pharmaceuticals Ltd Bangalore
- Tramadol obtained form M/S Ontop Pharmaceuticals Ltd Bangalore
- Methanol obtained from S D Fine Chemicals, Mumbai.
- Commercially available marketed dosage forms
- Zerodol-SR manufactured by TIL healthcare pvt Ltd., it contains 325 mg of paracetamol, 100 mg of Aceclofenac and 37.5 mg of Tramadol.
- HIFENAC-MR manufactured by TIL healthcare pvt Ltd. .it contains 325 mg of paracetamol,100 mg of Aceclofenac and 37.5 mg of Tramadol.
- UV-VIS Spectrophotometer SYSTRONICS-2201 was used for making all the spectral measurements.

4.1.2. Preparation of Standard Solution of Paracetamol, Aceclofenac and Tramadol

100 mg of Paracetamol, 25mg of Aceclofenac and 10 mg of Tramadol was weighed accurately, separately transferred into 50 ml Standard volumetric flask, dissolved and the volume was made up to the mark with methanol. (Ia, Ib and Ic respectively).

The 1ml of above three solutions carefully pippeted out and transfered separately into three 10 ml standard volumetric flask and the volume was made up to 10 ml at each flask with methanol. The resulting solution was having a concentration of 200, 50 and 20 µg/ml of Paracetamol, Aceclofenac and Tramadol respectively (solution IIa, IIb and IIc).

1ml of above three solutions was pippeted out separately into three 10 ml standard volumetric flask and the volume was made up to 10 ml at each flask with methanol. The resulting solution had the concentration of 20, 5, and 2 µg/ml of Paracetamol, Aceclofenac and Tramadol respectively (solution IIIa, IIIb and IIIc)

4.1.3. Spectral Characteristics of Paracetamol, Aceclofenac and Tramadol

After enabling the initial adjustments and Blank correction using methanol the solution IIIa, IIIb and IIIc was scanned separately in the UV region ranging form 200 nm to 400 nm. The obtained UVspectrum was showing maximum absorption for Paracetamol at 243nm, for Aceclofenac at 273nm and for Tramadol at 280nm which is shown in the Fig. 6,7and 8

4.1.4. Determination of Beer's law range

1 ml to 5 ml of solution was accurately pipetted out from IIa,IIb andIIc separately into five 10 ml standard volumetric flask, and the volume was made up to mark with methanol. The absorbance of each solution was measured at 243nm for Paracetamol, 273nm for Aceclofenac, and 280nm for Tramadol, against the reagent blank. The readings were plotted by taking concentration in X- axis and absorbance in y-axis as shown in the (Fig. 9,10and11) Beer's law was obeyed in the concentration in the range of Paracetamol is 20-100µg/ml, Aceclofenac 5-25µg/ml and Tramadol 2-10µg/ml.

4.1.5. Preparation and scanning of mixed standard solution

100 mg of Paracetamol, 25mg of Aceclofenac and 10 mg of Tramadol was weighed accurately and transferred into 50 ml Standard volumetric flask, dissolved and the volume was made up with methanol.(solution A).Accurately pippeted out 1ml of solution A into 10 ml standard volumetric flask and the volume was made up to 10 ml with methanol. The resulting solution had a concentration of 200, 50 and 20 µg/ml of Paracetamol, Aceclofenac and Tramadol respectively (solution B).

1ml, 2ml, 3ml, 4ml and 5ml of solution B was accurately pippeted out into five 10 ml standard volumetric flask respectively, and the volume was made up to 10 ml with methanol. The resulting solution had a concentration of 20-100 µg/ml of Paracetamol, 5-25µg/ml of Aceclofenac and 2-10µg/ml of Tramadol respectively .The absorbance of each solution was measured Paracetamol at 243 nm, Aceclofenac at 273 nm and Tramadol at 280 nm respectively. The data of absorbance versus drug concentration were treated by linear least square regression analysis to obtained

calibration graphs was shown in Fig. 9,10and11 and the values are written by Table 2, 3 and 4

4.1.6. LOD and LOQ

LOD and LOQ were calculated from the data obtained from the linearity studies. The slope of the linearity plot was determined. For each of the six replicated determinations-intercept was calculated and the standard deviation of y-intercept was computed. From the values LOD and LOQ were calculated as follows

$$\frac{3.3\,\sigma}{S} \quad \text{and} \quad \frac{10\,\sigma}{S}$$

Where, σ is the standard deviation of the intercepts

S is the average of slope.

4.1.7. Analysis of tablet formulation

Twenty tablets containing each of 325mg Paracetamol, 100 mg of Aceclofenac and 37.5 mg of Tramadol were accurately weighed and finely powdered in a glass mortar. The weight equivalent to 325mg Paracetamol, 100 mg of Aceclofenac and 37.5 mg of Tramadol was accurately weighed and transferred to a 100 ml standard volumetric flask. 40 ml of methanol was added and swirled gently for a period of 10 min. The clear supernatant solution was then transfered to 100ml standard volumetric flask through a whatmann No 1 filter paper. The residue was further extracted twice with 20 ml each of methanol and passed through the same filter paper and the volume was made up to 100ml with methanol. The

resulting solution had a concentration of 3.25 mg, 1mg and 0.375mg/ml of Paracetamol, Aceclofenac and Tramadol. (solution 1).

1 ml of the above solution was accurately pippeted into a 10 ml standard volumetric flask and made up to volume with methanol. The final solution had a concentration of 325 µg/ ml, 100µg/ml and 37.5µg/mlof Paracetamol, Aceclofenac and Tramadol respectively (solution 2)

Accurately pippeted out 1ml of solution B into 10ml standard volumetric flask and the volume was made up using methanol to obtain concentration of 32.5 µg/ml of Paracetamol, 10 µg/ml of Aceclofenac and 3.75 µg/ml of Tramadol respectively. The absorbance of this solution was measured at 243nm, 273nm and 280nm for Paracetamol, Aceclofenac and Tramadol respectively the concentration of each of drug was calculated using the regression equation at the particular calibration curve

The values are shown in Table 6 and 7.

4.1.8. Accuracy

Accuracy of the proposed method was performed by recovery technique. for this known quantities of Paracetamol, Aceclofenac and Tramadol were mixed with definite amount of pre analyzed formulations and the mixture were analysed.the total amount of Paracetamol, Aceclofenac and Tramadol was determined by using the proposed method and the amount of added drug was calculated by the difference.

$$\frac{\text{Total amount found by recovery-Actual amount found}}{\text{Amount of drug added}} \times 100$$

4.1.9. Precision

Precession was measured in terms of repeatability, which was determined by sufficient no. of aliquots of homogeneous sample and its %RSD was calculated.

4.1.10. Ruggedness and Robustness

Ruggedness was determined by changing laboratory studies. Robustness was determined by changing the analyst and % RSD was calculated.

Method 4.2. RP- HPLC method

4.2.1. Materials

The Chemicals and reagents used for experimental work are as follows

- Paracetamol obtained from M/S Biocon Ltd., Bangalore
- Aceclofenac obtained form M/S Biocon Ltd Bangalore
- Tramadol obtained form M/S Biocon Ltd .Bangalore
- HPLC water from Merck Specialities Private Ltd, Mumbai
- Sodium dihydrogen o-phosphate HPLC grade obtained from SD Fine Chemicals, Mumbai
- Acetonitrile HPLC grade was obtained from Qualigens Mumbai.
- Methanol HPLC grade was obtained from Qualigens Mumbai.
- Commercially available Pharmaceutical dosage form

- **ZERODOL-SR** manufactured by TIL healthcare pvt Ltd., it contains 325 mg of paracetamol, 100 mg of Aceclofenac and 37.5 mg of Tramadol.

- **HIFENAC-MR** manufactured by TIL healthcare pvt Ltd .it contains 325 mg of paracetamol,100 mg of Aceclofenac and 37.5 mg of Tramadol.

- High performance liquid chromatography SHIMADZU 1100 series equipped with isocratic pump, universal injector (Rheodyne) with injection volume of 20 mcL.

- Ultraviolet visible detector (MWD) and LC-software, a ZORBAZ Hypersil C_{18} ODS column (150mm x 4.6 mm i.d. 5 mcm particle size) forms the stationary phase, A calibrated electronic single Pan balance (Mettler AE 160)

4.2.2. OPTIMIZED CHROMATOGRAPHIC CONDITIONS:

S.No.	Parameter	Optimized condition
1	Chromatograph	SHIMADZU-HPLC
2..	Column	ZORBAZ Hypersil C_{18} -ODS column (150mm x 4.6 mm)
3.	Mobile phase	Methanol:Buffer:Water:Acetonitrile(85:10:25:5)
4.	Flow rate	1 ml/min
5.	Detection	UV at 276 nm
6.	Injection volume	20µl
7.	Temperature	Ambient

Table-1

4.2.3. Preparation of Mobile phase

The Phosphate buffer was prepared by dissolving 3.90 gms of sodium dihydrogen ortho-phosphate in 1000 mL volumetric flask, dissolved in sufficient volume of triple distilled water. And the final volume was made up with the same solvent. The resulting solution was sonicated for ten minutes and the pH was adjusted to 5.0± 0.2 by adding orthophosphoric acid. The mobile phase ratio was methanol: buffer: water: Acetonitrile (85:10:25:5) the prepared mobile phase was filtered through 0.22µm and degassing.

4.2.4. Preparation standard mixed solution of Paracetamol, Aceclofenac and Tramadol

The standard stock solution of Paracetamol, Aceclofenac and Tramadol were prepared by dissolving 100 mg of Paracetamol, 25 mg of Aceclofenac and 10 mg of Tramadol in 50 mL of mobile phase.(Solution A) The above solution of 1-5 ml was taken separately into five 10 ml standard volumetric flask respectively and the solution was having the final concentration of 200, 400, 600, 800 and 1000 µg/mL of Paracetamol, 50,100,150,200,and 250 µg/mL of Aceclofenac and 20, 40, 60, 80 and 100 µg/ml of Tramadol.(solutionB1-B5)The peaks were shown in the Fig 14(a),14(b),14(c),14(d)and14(e).

4.2.5. Preparation of sample solution

Twenty tablets of marketed samples (ZERODOL-SR and HIFENAC-MR) were separately weighed and made to a fine powder. The tablet powder equivalent to 325 mg of Paracetamol, 100 mg of Aceclofenac and 37.5 mg of Tramadol were

transferred to a conical flask and extracted with methanol. The extract was filtered through Watt man filter paperNo.1 and the washings were pooled, transfered to a 100 mL standard volumetric flask and the final volume was made upto100 mL with mobile phase. From that solution 2 mL was diluted to 10 mL .Which contains 650 µg/mL of Paracetamol, 200 µg/mL of Aceclofenac and 75 µg/mL of Tramadol. (T1 and T2).The values are shown in Table 13 and 14.The peaks are shown in Fig 11 and 12.

4.2.6. Preparation of calibration curves

The solutions of B1-B5 were injected separately under optimized chromatographic conditions. Peak area was recorded for all the peaks. (Table 8,9 and 10).The graphs (Fig.16,17 and 18**)** of peak area versus the respective concentrations of Paracetamol, Aceclofenac and Tramadol were found to be linear in the range of 200 µg /ml – 1000 µg /ml, 50 µg /ml– 250 µg /ml, and 20 µg /ml - 100 mcg/ml respectively.

4.2.7 Linearity Data of Paracetamol, Aceclofenac and Tramadol

On the observation of (Table 11,12 & 13 and The Fig. 16,17 and 18) peak area versus the respective concentrations of Paracetamol, Aceclofenac and Tramadol were found to be linear in the range of 200 µg /ml – 1000 µg /ml, 50 µg /ml – 250 µg /ml, and 20 µg /ml - 100 µg /ml respectively. The Response factor (Amount / Area) for each concentration of Paracetamol, Aceclofenac and Tramadol were found to be constant.

Correlation coefficient (r) is calculated applying the formula (Kari Pearson's correlation coefficient Method) –

$$r = \frac{n\Sigma XY - \Sigma X\,\Sigma Y}{[\{\, n\,\Sigma x^2 - (\Sigma X)^2 \,\}\,\{n\,\Sigma Y^2 - (\Sigma Y)^2)]^{1/2}}$$

Slope (a) of straight line is calculated by formula –

$$a = \frac{n\Sigma XY - \Sigma X\,\Sigma Y}{n\,\Sigma x^2 - (\Sigma X)^2}$$

Intercept (b) of straight line is calculated by formula –

$$B = Y - a\,X$$

Where,

N = no of observation = 6,

Y is arithmetic mean value of Y

and X is arithmetic mean value of X,

a-slope of Straight line as mentioned above and substituting the arithmetic mean value of Y and X.

The linear regression equation for a straight line was obtained by substituting the values of slope (a) and intercept (b) in the following equation of Y on X given as

$$Y = aX + b.$$

Where X and Y indicates x – axis and Y 0 axis values, we got the equation as Y=15.574X + 274.37, Y=31.457X + 21.036and Y=97.747X + 297.08 which indicates the straight line (linearity) equation for the drug Paracetamol, Aceclofenac and Tramadol respectively. The value of Correlation coefficient (r), slope (a) and intercept (b) for these three drugs were shown in Table 11.

4.2.8. LOD and LOQ

LOD and LOQ were calculated from the data obtained from the linearity studies. The slope of the linearity plot was determined. For each of the six replicated Determinations-intercept was calculated and the standard deviation of y-intercept was computed. From the values LOD and LOQ were calculated as follows

$$\frac{3.3\,\sigma}{S} \quad \text{and} \quad \frac{10\,\sigma}{S}$$

Where σ is the standard deviation of the response,

S is the average of slope.

4.2.9. Precision

Precision was measured in terms of repeatability, which was determined by sufficient no. of aliquots of homogeneous sample and its %RSD was calculated.

4.3.0. Accuracy

Accuracy of the proposed method was performed by recovery technique. for this known quantities of Paracetamol, Aceclofenac and Tramadol were mixed with definite amount of pre analyzed formulations and the mixture were analysed.

The total amount of Paracetamol, Aceclofenac and Tramadol was determined by using the proposed method and the amount of added drug was calculated by the difference.

$$\frac{\text{Total amount found by recovery} - \text{Actal amount found}}{\text{Amount of drug added}} \times 100$$

4.3.1. Ruggedness and Robustness

Ruggedness was determined by changing laboratory studies. Robustness was determined by changing the analyst and % RSD were calculated.

4.3.2. System suitability tests

As per USP-26, system suitability tests were carried out on freshly prepared standard stock solutions of Paracetamol, Aceclofenac and Tramadol. 20mcl of each drug were injected into the chromatograph under the optimized chromatographic conditions. Parameters that were studies to evaluate the suitability of the system. The values of system suitability of the system. The values of system suitability tests were shown in Table 12.

5. RESULTS AND DISCUSSION

The marketed products ZERODOL-SR and HIFENAC-MR were analyzed to develop new methods for simultaneous estimation of Paracetamol, Aceclofenac and Tramadol in combined dosage form. The results obtained by the proposed method were found to be satisfactory.

The spectroscopic method obeyed Beer's Law in low concentration, which is an advantage in routine analysis. The results obtained by the different methods are mentioned in Table No.15and 16.

Absorption spectra of Paracetamol at 243 nm

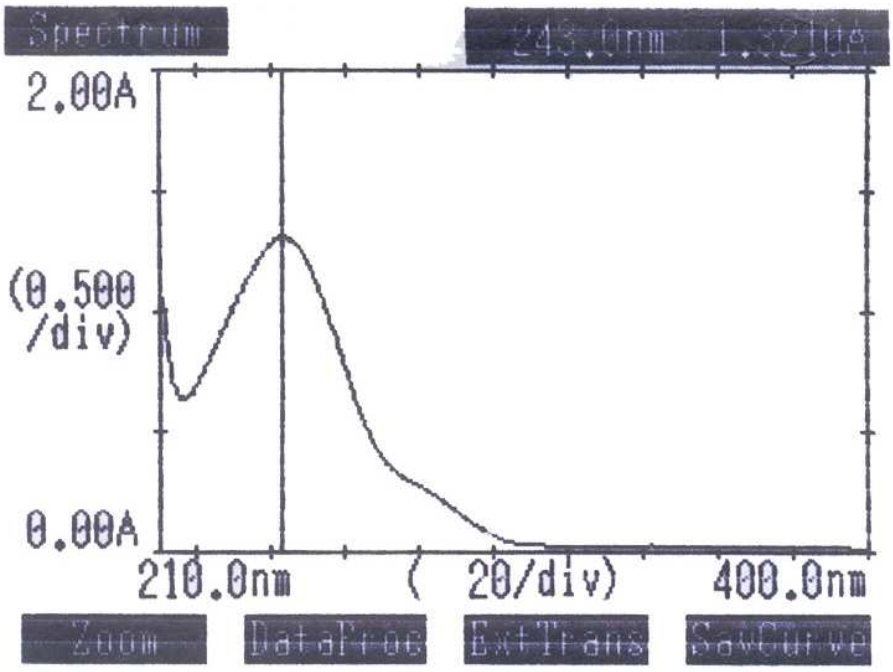


Fig: 6

Absorption spectra of Aceclofenac at 273 nm

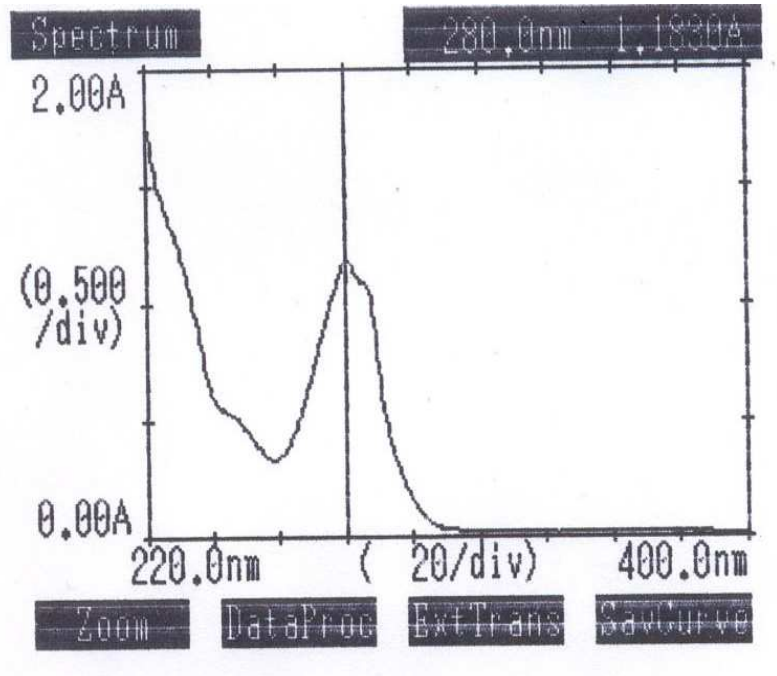


Fig: 7

Absorption spectra of Tramadol at 280 nm

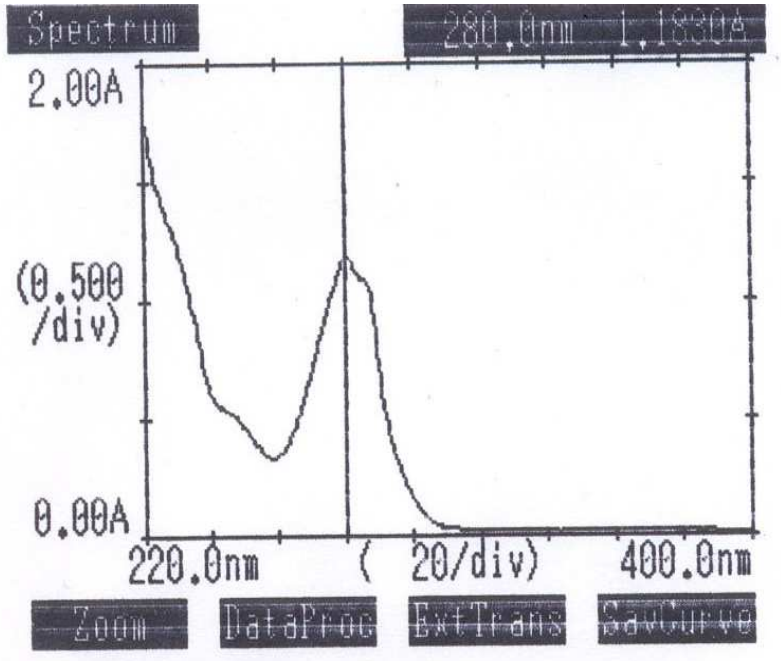


Fig: 8

Beer's law plot for Paracetamol at 243nm

S.NO	Concentration in mcg/ml	Absorbance
1	20	0.253
2	40	0.462
3	60	0.685
4	80	0.894
5	100	1.142

Table-2

Linearity of Paracetamol at 243 nm

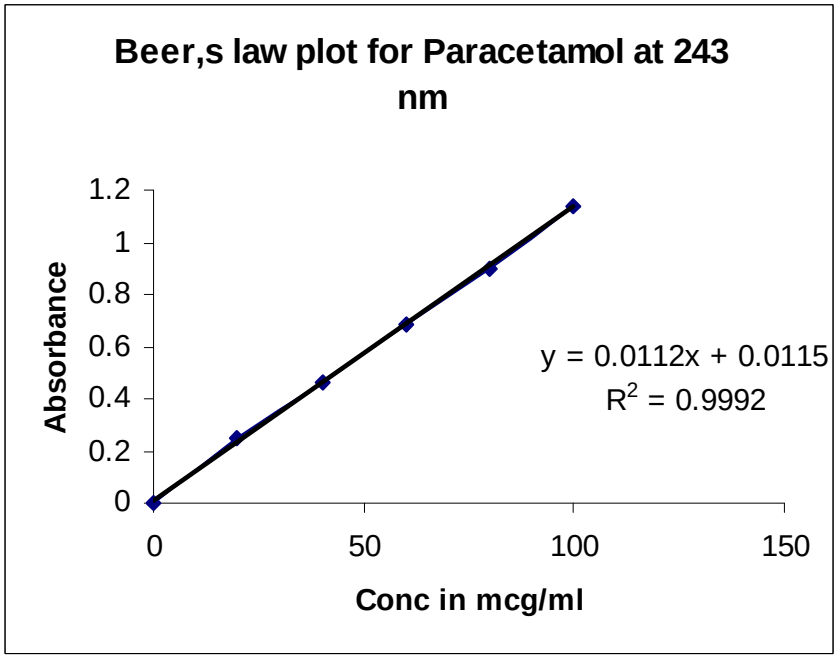


Fig: 9

Beer's law plot for Aceclofenac at 273nm

S.NO.	Concentration in mcg/ml	Absorbance
1	5	0.189
2	10	0.354
3	15	0.543
4	20	0.723
5	25	0.864

Table-3

Linearity of Aceclofenac at 273 nm

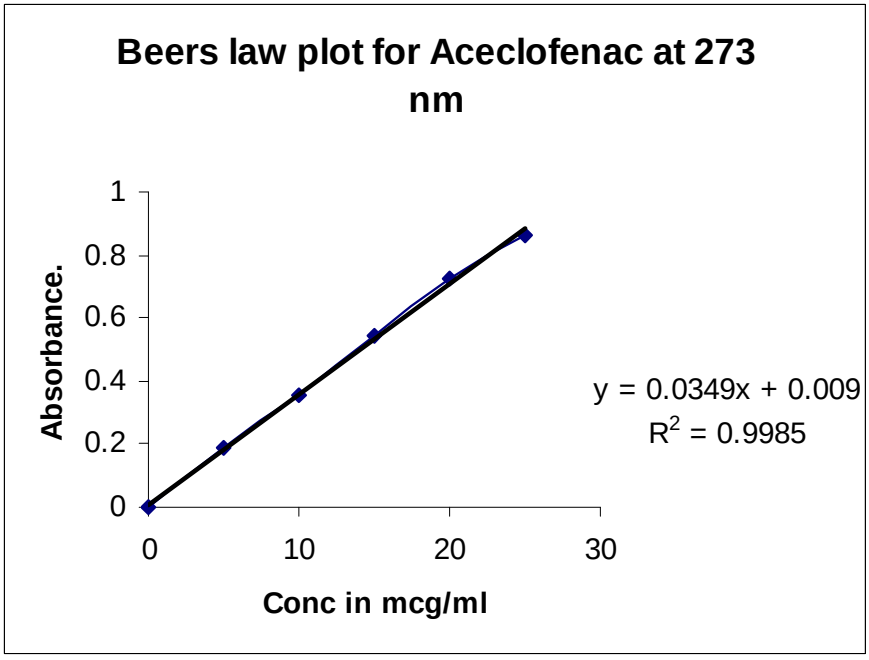


Fig: 10

Beer's law plot for Tramadol at 280nm

S.NO	Concentration in mcg/ml	Absorbance
1	2	0.167
2	4	0.342
3	6	0.497
4	8	0.694
5	10	0.831

Table-4

Linearity of Tramadol at 280 nm

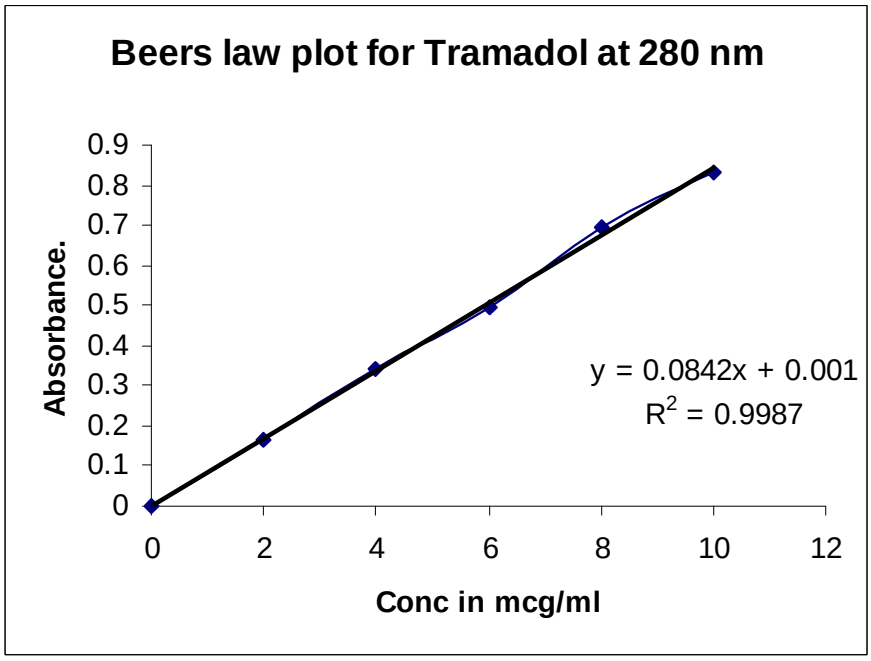


Fig: 11

Table-5 : Optical characters and other parameters.

Parameters	Paracetamol	Aceclofenac	Tramadol
λ_{max} (nm)	243nm	273nm	280nm
Beer's law(mcg/ml)	20-100 mcg/ml	5-25 mcg/ml	2-10 mcg/ml
Slope	0.0112	0.0349	0.0842
Intercept	0.0115	0.009	0.001
Regression equation	Y=0.0112x+0.0115	Y=0.0349x+0.009	Y=0.0842x+0.001
Correlation co-efficient (R^2)	0.9992	0.9985	0.9987
LOD	4.5	1.3	0.6
LOQ	13.5	3.9	1.80
$E^{1\%}_{1cm}$	115.5	108.2	98.5

Table-6 : Results of formulation values (ZERODOL-SR)

PARAMETERS	VALUES		
	Paracetamol	Aceclofenac	Tramadol
%Content ± %RSD*	99.32 + 0.97	98.45 + 0.93	99.82 + 0.89
Accuracy(%Recovery) ± %RSD*	99.26 + 0.89	99.84 ± 0.91	98.76 ±1.01
Precision (i) Intra day ± %RSD* (ii) Inter day ± %RSD*	 99.17 ± 0.99 98.76 ± 0.83	 99.78 ± 0.85 98.65 ± 0.76	 98.97 ±0.97 98.87 ± 0.68
Ruggedness ± %RSD*	99.45 ± 0.97	99.25 ±0.84	98.75 ±0.66
Robustness ± %RSD*	99.52 ± 1.01	99.21± 0.78	99.52 ± 0.78

*Average of six determinations, %RSD indicates Relative standard deviation

Table-7 : Results of formulation values (HIFENAC-MR)

PARAMETERS	VALUES		
	Paracetamol	**Aceclofenac**	**Tramadol**
%Content ± %RSD*	99.49 ± 0.98	99.31 ± 1.15	99.81 ± 1.01
Accuracy(%Recovery)±%RSD*	98.38 ± 0.84	99.59 ± 0.91	99.82 ± 0.95
Precision (iii) Intra day ± %RSD* (iv) Inter day ± %RSD*	 99.70 ± 0.94 98.83 ± 0.87	 99.89 ± 0.99 97.97 ± 0.81	 99.09 ± 1.10 98.92 ± 0.84
Ruggedness ± %RSD*	98.64 ± 0.87	99.79 ± 0.92	99.45 + 0.82
Robustness ± %RSD*	99.12±0.96	99.81 ± 0.88	99.27 ±0.96

*Average of six determinations, %RSD indicates Relative standard deviations

Chromatogram of Paracetamol, Aceclofenac and Tramadol (ZERODOL-SR)

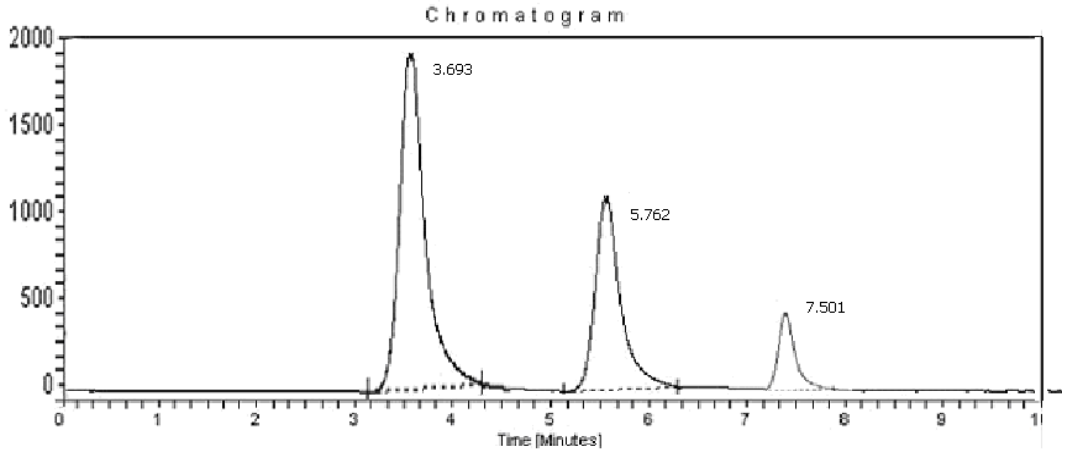


Fig: 12

Peak No	Retn.Time	Area
1. Paracetamol	3.693	3456.69
2. Aceclofenac	5.672	1628.39
3. Tramadol	7.501	2332.96

Chromatogram of Paracetamol, Aceclofenac and Tramadol (HIFENAC-MR)

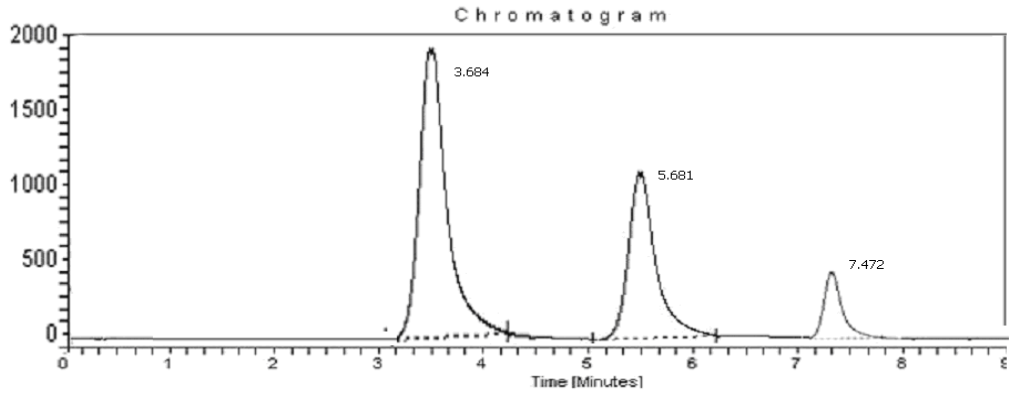


Fig: 13

Peak No	Retn.Time	Area
1. Paracetamol	3.684	3456.23
2. Aceclofenac	5.681	1629.31
3. Tramadol	7.472	2334.38

Chromatogram of Paracetamol, Aceclofenac and Tramadol standard mixture (1)

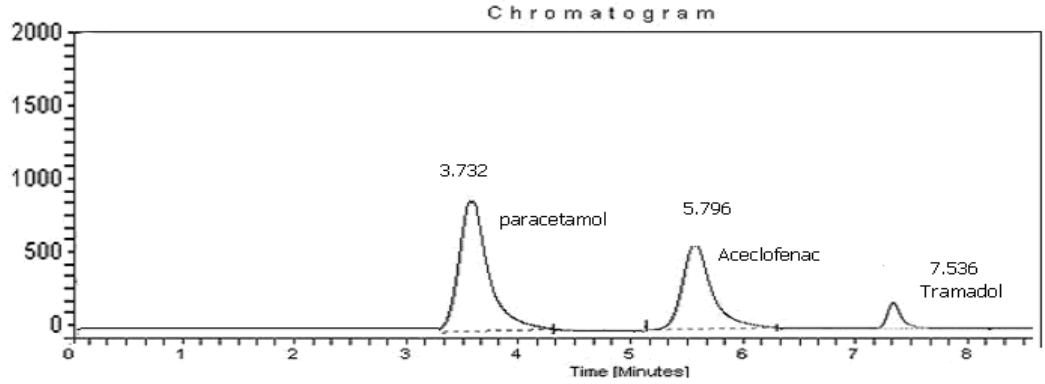


Fig: 14(a)

Peak No	Retn.Time	Area
1. Paracetamol	3.732	3456.23
2. Aceclofenac	5.796	1625.25
3. Tramadol	7.536	2332.24

Chromatogram of Paracetamol, Aceclofenac and Tramadol standard mixture (2)

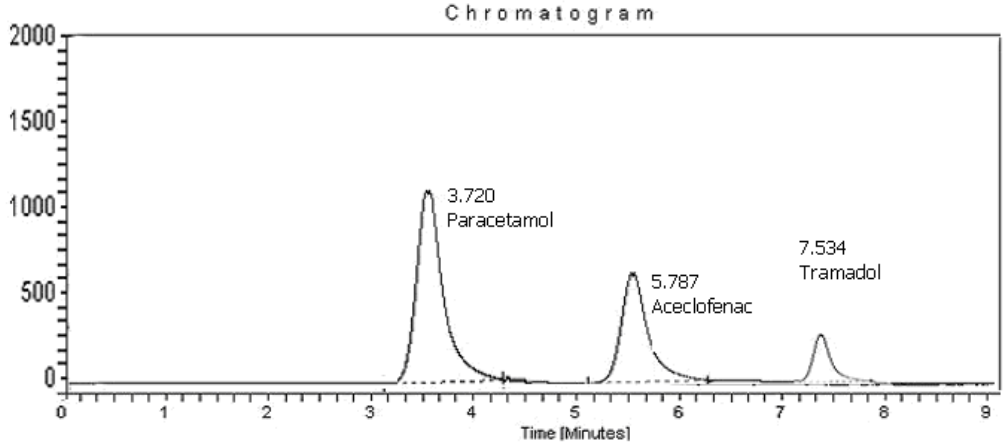


Fig: 14(b)

Peak No	Retn.Time	Area
1. Paracetamol	3.720	7123.21
2. Aceclofenac	5.787	3256.78
3. Tramadol	7.536	4556.36

Chromatogram of Paracetamol, Aceclofenac and Tramadol standard mixture (3)

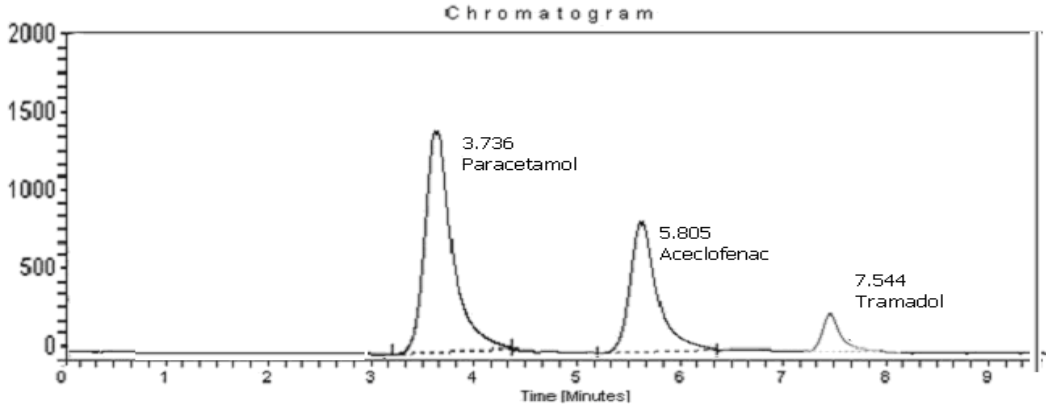


Fig: 14(c)

Peak No.	Retn.Time	Area
1. Paracetamol	3.736	9126.21
2. Aceclofenac	5.805	4632.54
3. Tramadol	7.544	6435.25

Chromatogram of Paracetamol, Aceclofenac and Tramadol standard mixture (4)

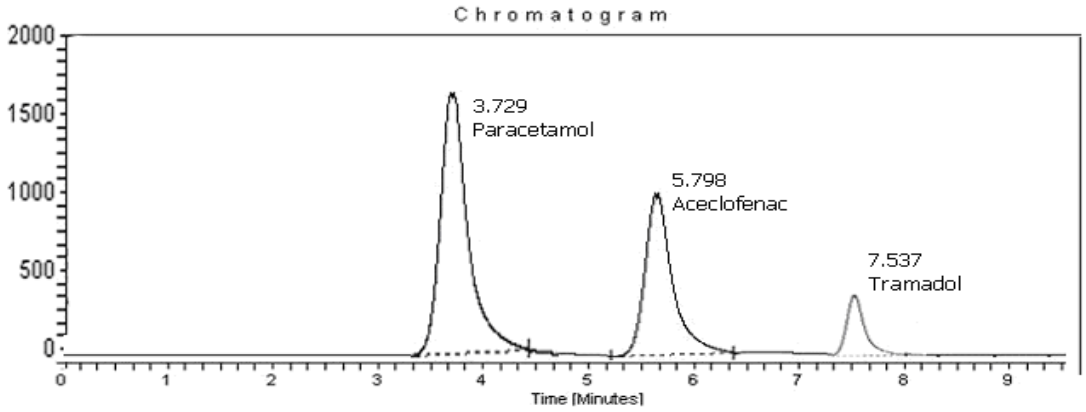


Fig: 14(d)

Peak No	Retn.Time	Area
1. Paracetamol	3.729	12963.35
2. Aceclofenac	5.798	6235.98
3. Tramadol	7.537	7986.32

Chromatogram of Paracetamol, Aceclofenac and Tramadol standard mixture (5)

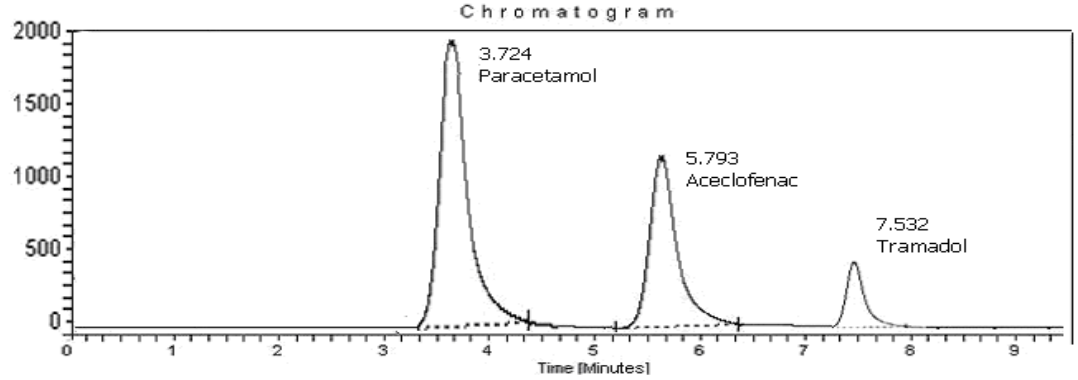


Fig: 14(e)

Peak No	Retn.Time	Area
1. Paracetamol	3.724	15698.32
2. Aceclofenac	5.793	7968.31
3. Tramadol	7.532	9896.32

Chromatogram of Paracetamol, Aceclofenac and Tramadol standard mixture

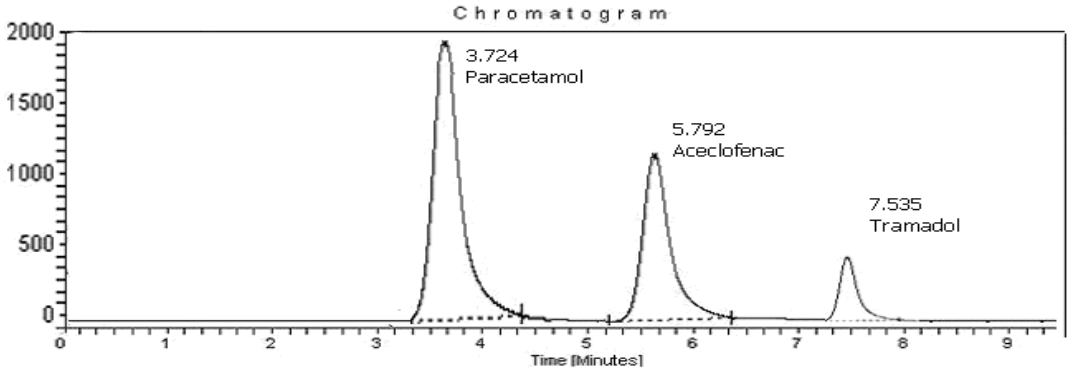


Fig: 15

Peak No	Retn.Time	Area
1. Paracetamol	3.724	3456.38
2. Aceclofenac	5.792	1625.39
3. Tramadol	7.535	2338.96

Table No-8 : Linearity Data of Paracetamol

S. No.	Conc. in mcg/ml	Peak Area
1	200	3456.23
2	400	7123.21
3	600	9126.21
4	800	12963.35
5	1000	15698.32

Linearity of Paracetamol

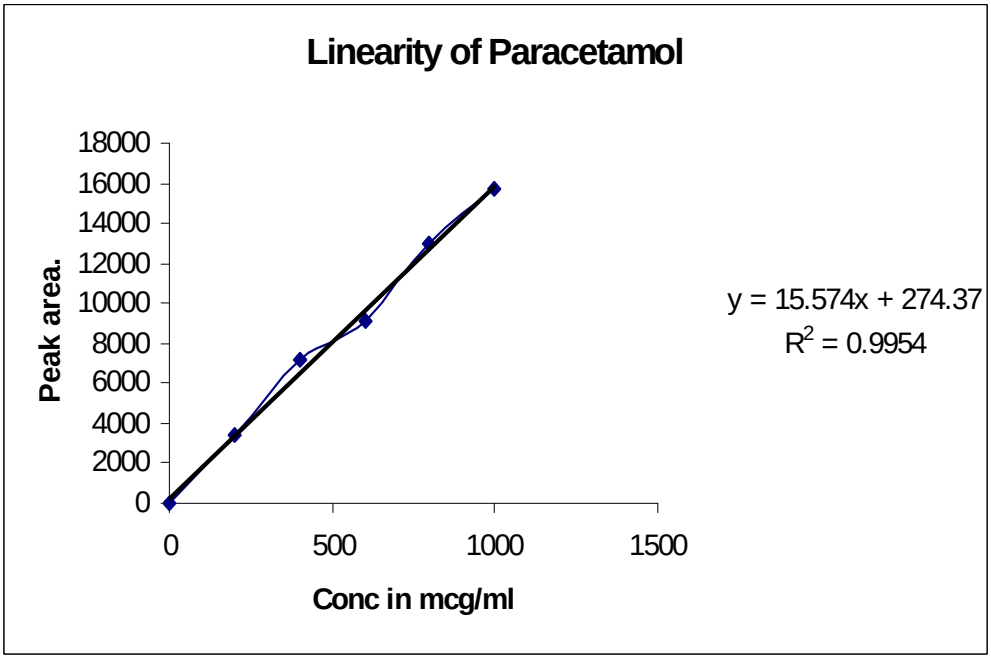


Fig: 16

Table No-9 : Linearity Data of Aceclofenac

S. No.	Conc. in mcg/ml	Peak Area
1	50	1625.25
2	100	3256.78
3	150	4632.54
4	200	6235.98
5	250	7968.31

Linearity of Aceclofenac

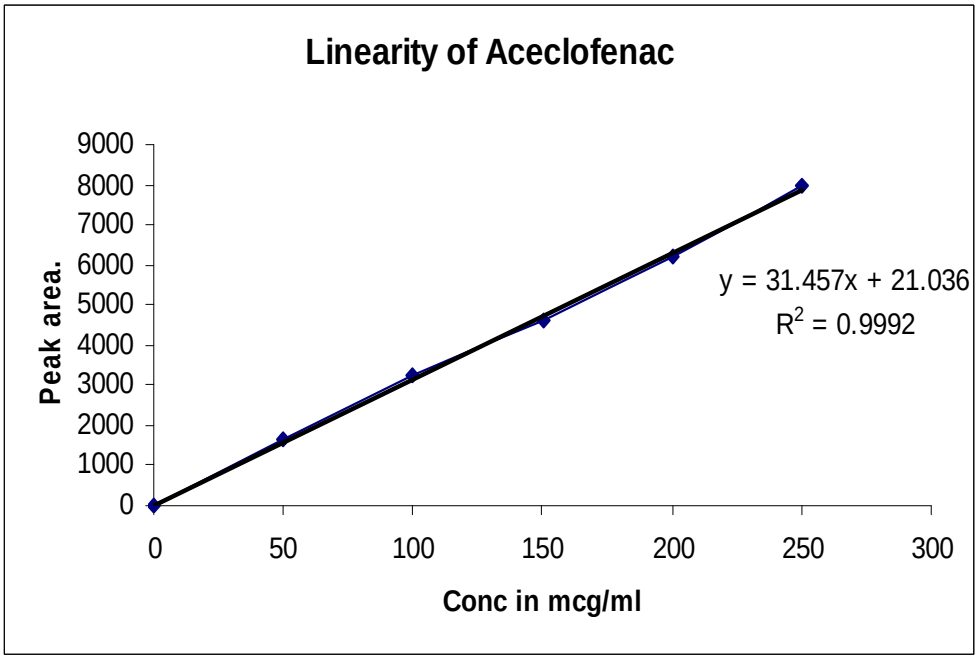


Fig: 17

Table No-10 : Linearity Data of Tramadol

S. No.	Conc. in mcg/ml	Peak Area
1	20	2332.24
2	40	4556.36
3	60	6435.25
4	80	7986.32
5	100	9896.32

Linearity of Tramadol

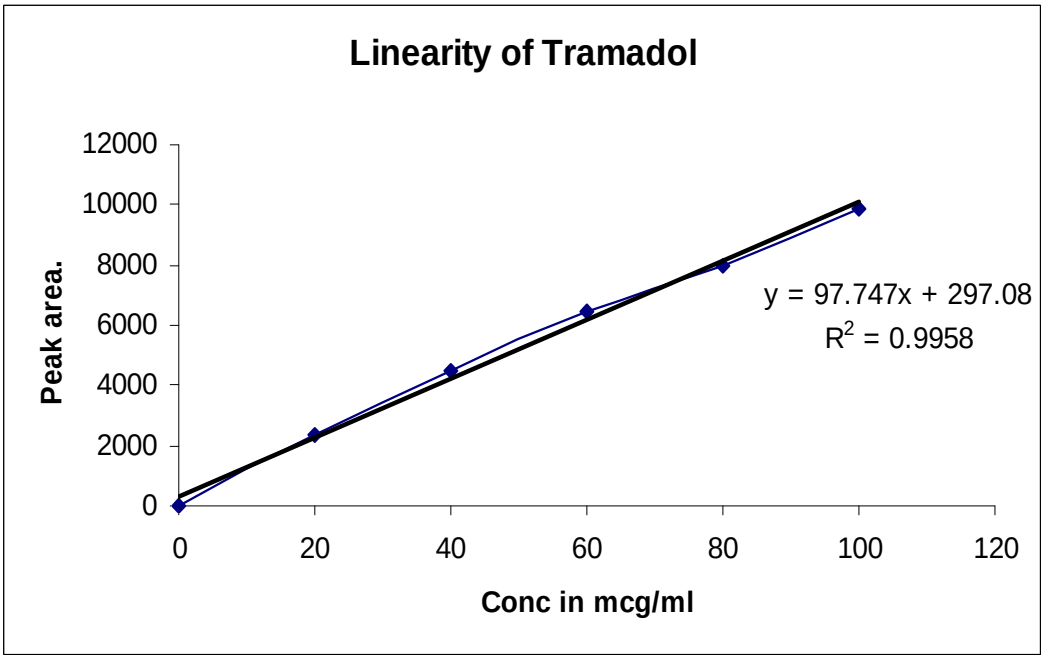


Fig: 18

Table No-11 : Optical characters and other parameters.

Parameters	**Paracetamol**	**Aceclofenac**	**Tramadol**
Linearity(mcg/ml)	200-1000	50-250	20-100
Regression equation	Y=15.574x+274.37	Y=31.457x+21.036	Y=97.747x+297.08
Correlation coefficient(R^2)	0.9954	0.9992	0.9958
Slope	15.574	31.457	97.747
Intercept	274.37	21.036	297.08
Standard Deviation(SD)	118.60	28.379	35.419
RSD (%.)	0.9968	0.9687	0.9987
LOD(µg/ml)	0.2786	0.1128	0.1284
LOQ(µg/ml)	0.152	0.242	0.057

Table No-12 : System Suitability parameters

S.No	Parameters	Paracetamol	Aceclofenac	Tramadol
1	No. of theoretical plats	1461.736	2238.478	2413.092
2	Resolution	1.14	4.58	7.29
3	Retention time	3.724	5.792	7.535
4	R.S.D. for Retention time	0.058	0.45	0.335
5	R.S.D. for Peak Area	0.085	0.108	0.117
6	Tailing factors/asymmetry factors	1.3	1.28	1.28

Table-13 : Results of formulation values (ZERODOL-SR)

PARAMETERS	VALUES		
	Paracetamol(325mg)	Aceclofenac(100mg)	Tramadol(37.5mg)
%Content ± %RSD*	99.58 ± %0.872	99.67 ± %0.861	99.21 ± %0.912
Accuracy (%Recovery) ± %RSD*	97.98 ± %0.865	98.27 ± %0.933	98.61 ± %0.903
Precission			
(i)Intra day ± %RSD*	99.13 ± %0.876	99.36 ± %0.914	99.82 ± %0.890
(ii)Inter day ± %RSD*	98.01 ± %0.829	97.87 ± %0.856	98.18 ± %0.868
Ruggedness ± %RSD*	99.13 ± %0.887	99.87 ± %0.916	99.56 ± %0.909
Robustness ± %RSD*	97.9 ± %0.865	97.02 ± %0.855	98.16 ± %0.899

*Average of six determinations, %RSD indicates Relative standard deviation

Table-14 : Results of formulation values (HIFENAC-MR)

PARAMETERS	VALUES		
	Paracetamol	**Aceclofenac**	**Tramadol**
%Content ± %RSD*	99.02 ±%0.923	98.91 ±%0.891	99.32 ±%0.936
Accuracy (%Recovery) ±%RSD*	99.45 ±%0.954	98.72 ±%0.912	99.07 ±%0.941
Precision			
(i)Intra day ± %RSD*	99.78 ±%0.974	98.38 ±%0.902	99.21 ±%0.909
(ii)Inter day ± %RSD*	99.06 ±%0.899	98.03 ±%0.895	99.32 ±%0.913
Ruggedness ± %RSD*	99.89 ±%0.983	98.98 ±%0.993	99.67 ±%0.984
Robustness ± %RSD*	99.76 ±%0.971	99.02 ±%0.893	97.95 ±%0.801

*Average of six determinations, %RSD indicates Relative standard deviation

Table No. 15: Result of analysis of marketed dosage forms by U.V Method

UV METHOD		VALUES
Wave length	Paracetamol	243nm
	Aceclofenac	273nm
	Tramadol	280nm
Beer's range µg/ml	Paracetamol	20-100
	Aceclofenac	5-25
	Tramadol	2-10
R^2	Paracetamol	0.9992
	Aceclofenac	0.9985
	Tramadol	0.9987
Slope	Paracetamol	0.0112
	Aceclofenac	0.0349
	Tramadol	0.0842
LOD µg/ml	Paracetamol	4.5
	Aceclofenac	1.3
	Tramadol	0.6
LOQµg/ml	Paracetamol	13.5
	Aceclofenac	3.9
	Tramadol	1.84
RSD_1	Paracetamol	3.499
	Aceclofenac	1.634
	Tramadol	4.491
RSD_2	Paracetamol	3.514
	Aceclofenac	0.439
	Tramadol	1.612

Table No. 16 : Result of analysis of marketed dosage forms by HPLC Method

HPLC METHOD		VALUES
R^2	Paracetamol	0.9954
	Aceclofenac	0.9992
	Tramadol	0.9958
LOD µg/ml	Paracetamol	0.2963
	Aceclofenac	0.0224
	Tramadol	0.124
LOQµg/ml	Paracetamol	0.1757
	Aceclofenac	0.055
	Tramadol	0.147
RSD_1	Paracetamol	3.499
	Aceclofenac	1.634
	Tramadol	4.491
RSD_2	Paracetamol	3.514
	Aceclofenac	0.439
	Tramadol	1.612

DISCUSSION

UV Spectroscopic method

Specific accurate precise and simple UV spectroscopic methods were developed for simultaneous determination of Paracetamol, Aceclofenac and Tramadol from their dosage form. The first method was based on Calibration curve method. In the proposed Calibration curve method, the signals were measured at 243nm, 273nm and 280nm corresponding to the absorbance maxima of Paracetamol, Aceclofenac and Tramadol in methanol respectively. The Concentration of each drug was obtained by using the Calibration curve. The method was validated statistically. Recovery study was performed to confirm the accuracy of the method. This method is suitable technique for the reliable analysis of commercial formulation containing combination of Paracetamol, Aceclofenac and Tramadol. Simplicity, sensitivity and rapidity of Calibration curve method render it suitable for routine analysis for of Paracetamol, Aceclofenac and Tramadol from their combination dosage forms.

In addition the above-proposed UV spectroscopic method was simple, easy to apply low cost, does not use polluting reagents and requires relatively inexpensive instruments. Then it may be considered as good alternative to the RP-HPLC method for simultaneous estimation of Paracetamol, Aceclofenac and Tramadol in dosage forms.

RP-HPLC method

A Reverse Phase High pressure liquid chromatographic method has been developed for simultaneous estimation of Paracetamol, Aceclofenac and Tramadol in its tablet form. A Hypersil C_{18} octadecyl silane (ODS) column (150mmx4.6 mm i.d., 5 mcm Particle size, 68% void volume) in isocratic mode, with mobile phase Methanol: phosphate buffer: Water: Acetonitrile. The flow rate was 1 ml per minute and effluent was monitored at 272mm. The approximate retention time for of Paracetamol, Aceclofenac and Tramadol were 3.724 min., 5.792 min and 7.535 min respectively. The linearity for Paracetamol, Aceclofenac and Tramadol was in the range of 200 mcg/ml – 1000 mcg/ml, 50mcg/ml –250 mcg/ml and 20mcg/ml –100 mcg/ml respectively. Quantity found for Paracetamol, Aceclofenac and Tramadol was 324.76 mg/av.wt, 99.86 mg/av.wt and 37.21 mg/av.wt. respectively. Percentage recoveries obtained for the drugs were 100.6%, 100.3% and 99.4% respectively. The proposed method is precise and rapid for simultaneous estimation of Paracetamol, Aceclofenac and Tramadol in tablet formulation.

Interference studies

The other active ingredients and common excipients present in the dosage forms of Paracetamol, Aceclofenac and Tramadol did not interfere, when added in the mentioned concentration ranges to the drug and estimated by the proposed methods. The methods reported here are found to be simple, sensitive.accurate, precise and economical and can be used in the determination of Paracetamol, Aceclofenac and Tramadol from pharmaceutical formulations in a routine manner.

6. SUMMARY AND CONCLUSION

Many of the effects of NSAIDs (Paracetamol and Aceclofenac) appear to be due to their inhibitory action on cyclo-oxygenase which is involved in the biosynthesis of prostaglandins and thromboxanes from arachidonic acid. Prostaglandins have an important role in the production of pain, inflammation and fever. Hence, they are useful in the symptomatic relief and painful and/or inflammatory conditions including rheumatic disorders such as rheumatoid arthritis, osteoarthritis and the spondyloarthropathies and also in peri-articular disorders and soft tissue rheumatism. Tramadol is a centrally acting analgesic and relieves pain by opoid as well as additional mechanisms.

However so far no spectrometric and RP-hplc methods for simultaneous estimation of these three drugs in the combined dosage form till date.

This research work has been taken up to develop and validate simple, sensitive and cost effective methods for the estimation of Paracetamol, Aceclofenac and Tramadol in a combined dosage form.

CONCLUSSION

The proposed methods are simple, rapid, and validated and can be used successfully for routine simultaneous estimation.

Capabilities of the two methods are complementary to each other. Hence they can be regarded as simple, specific and sensitive methods for the estimation of Paracetamol, Aceclofenac and Tramadol in combined dosage forms. The % RSD values of Accuracy, Precision, Ruggedness and Robustness values are within the range of 0.8 ± 2.

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